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Review Paper

CRISPR therapy towards an HIV cure

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Abstract

Tools based on RNA interference (RNAi) and the recently developed clustered regularly short palindromic repeats (CRISPR) system enable the selective modification of gene expression, which also makes them attractive therapeutic reagents for combating HIV infection and other infectious diseases. Several parallels can be drawn between the RNAi and CRISPR-Cas9 platforms. An ideal RNAi or CRISPR-Cas9 therapeutic strategy for treating infectious or genetic diseases should exhibit potency, high specificity and safety. However, therapeutic applications of RNAi and CRISPR-Cas9 have been challenged by several major limitations, some of which can be overcome by optimal design of the therapy or the design of improved reagents. In this review, we will discuss some advantages and limitations of anti-HIV strategies based on RNAi and CRISPR-Cas9 with a focus on the efficiency, specificity, off-target effects and delivery methods.

Key words: RNA interference; CRISPR-Cas; HIV; gene therapy; lentiviral vector; polymerase III promoter

HIV and AIDS

More than 30 years after the discovery of the human immunodeficiency virus type 1 (HIV-1) as the causative agent of the acquired immunodeficiency syndrome (AIDS), HIV remains one of the most serious infectious diseases in the world [1, 2]. Despite significant research efforts, there is still no vaccine available that protects against HIV infection. A combination of several drugs, known as combined antiretroviral therapy (cART), is currently used to keep HIV replication under control and to successfully treat HIV patients. Although cART can suppress viral replication, a cure is never achieved because the virus persists in some cells [3]. As a consequence, patients need to take the antiviral drugs for the rest of their life. Daily medication can create difficulties with adherence, and drug-associated side effects can occur over time [4]. Thus, the search for alternative strategies for combating HIV is warranted. An attractive approach concerns a gene therapy to deliver antiviral gene reagents that interfere with viral replication to cells that can be infected by HIV. Technologies to modify gene expression, either gene silencing at the RNA level by RNA interference (RNAi) or genome editing at the DNA level by the recently developed clustered regularly short palindromic repeats (CRISPR)-based tools offers new possibilities to inhibit HIV, a retrovirus that uses both RNA and DNA forms of genetic information [5–8]. We previously reviewed the antiviral RNAi approaches [9] and will now use this as background for a discussion of the more novel CRISPR-based anti-HIV strategies.

RNAi and CRISPR-Cas9 against HIV

RNAi provides a powerful tool to elucidate gene function by silencing the expression of a specific gene at the RNA level. The control of disease-associated genes makes RNAi an attractive choice for future therapeutics. The recently discovered CRISPR-Cas9 bacterial immune system can be repurposed to easily create gene mutations and replacements in the mammalian genome and has revolutionized the field of genome engineering and reinforced the field of gene therapy research. Both RNAi and CRISPR-Cas9 approaches enable the selective modification of gene expression and are therefore intriguing therapeutic reagents for combating HIV and other infectious diseases [5, 7, 8, 10]. Many parallels can be drawn between the RNAi and

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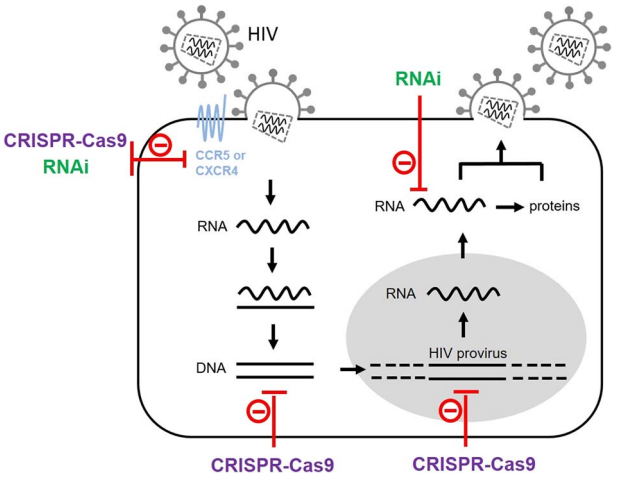

Figure 1. HIV targeting by RNAi or CRISPR-Cas9. HIV infects cells of the immune system, in particular the CD4-positive T cells. The HIV particle contains two genomic RNA copies. The virion attaches to the membrane of target T cells by binding to the CD4 and CCR5/CXCR4 receptors. Upon viral entry, the viral RNA genome is converted into double-stranded DNA (dsDNA) by the HIV reverse transcriptase. The resulting DNA is actively transported into the nucleus and integrated in the host cell genome. This integrated DNA or provirus uses the host cell transcription machinery to produce new viral RNAs, which serve as mRNA for protein production or as genomic RNAs that are packaged into new viral particles, which are released from the cell by budding. RNAi can target the RNA transcripts that encode the HIV receptors to block viral entry. In addition, RNAi can target the viral RNA produced during HIV replication. The receptor-encoding genes can be targeted by CRISPR-Cas9, which can also target the HIV dsDNA that is formed upon reverse transcription of the viral RNA and the integrated proviral DNA.

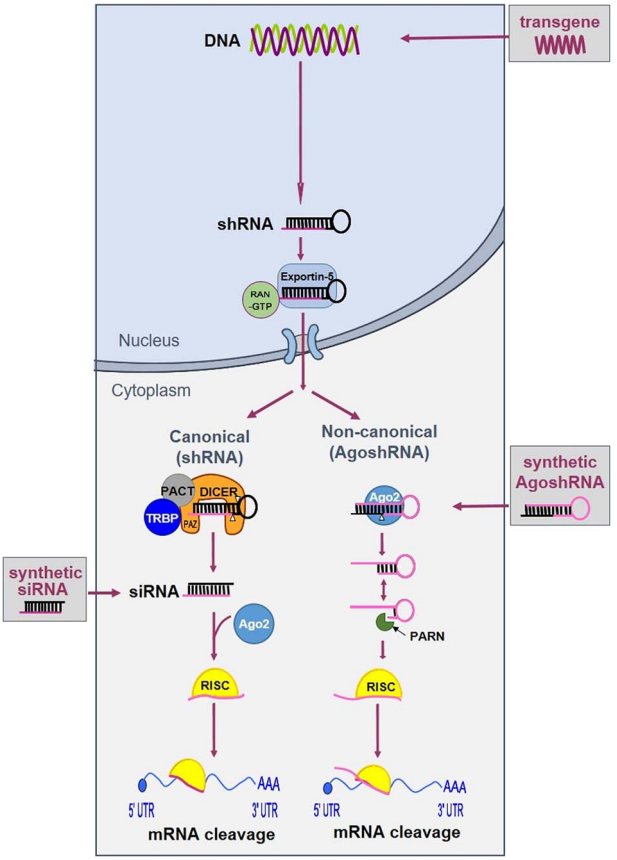

Figure 2. Schematic of two RNAi pathways available for shRNA processing. shRNA transcripts can be expressed from an episomal or stably integrated expression cassette. The shRNAs are then exported from the nucleus to the cytoplasm for processing: regular shRNAs by Dicer (left) and the shorter AgoshRNAs by Ago2 (right).

CRISPR-Cas9 platforms. A clinically ideal RNAi or CRISPR-Cas9 reagent for treating infectious or genetic diseases should exhibit potency, high specificity and safety. However, therapeutic applications of RNAi and CRISPR-Cas9 have been challenged by several major limitations, but some of these limitations can be overcome by the optimal design of the therapy or development of improved reagents. In this chapter, we will discuss advantages and limitations of RNAi and CRISPR-Cas9 with a focus on the efficiency, specificity, off-target effects and delivery methods.

The efficiency and sequence specificity of the RNAi and CRISPR-Cas9 systems make them attractive strategies to interrupt the expression of disease-associated genes or pathogenic viruses [6–8, 10]. As shown in Figure 1, RNAi and CRISPR-Cas9 can be used to target viral or host functions that play critical roles at during HIV replication. Many studies reported efficient suppression of HIV replication by these two approaches [3, 10–21]. A recent paper demonstrated that CRISPR treatment can eliminate infectious HIV in a subset of infected humanized mice [22]. Nevertheless, there are challenges that must be overcome before the RNAi and CRISPR-Cas therapeutics can fulfill their clinical potential. These issues include possible off-target effects, improvement of delivery strategies and approaches to reduce the risk of viral escape [8, 23].

RNAi

Efficiency:

Since the first application of RNAi in mammalian cells, the expression of shRNA molecules for targeted gene silencing has become a benchmark technology. The RNAi pathway can be triggered by artificial RNAi effectors that mimic the intermediates of miRNA processing, including natural small interfering RNAs (siRNAs) [24] and the man-made short hairpin RNAs (shRNAs) [25]. The latter—expressed from a transgene introduced in the cell—have the potential to achieve stable gene silencing. In Figure 2, we describe two possible processing routes for a shRNA substrate [26, 27]. A regular shRNA (left side) features a ~21-base pair (bp) stem with a 5–9-nucleotide (nt) loop and is processed by Dicer into a siRNA duplex of ~20 bp, of which one strand is preferentially loaded into the Argonaute 2 protein (Ago2) complex to form the RNA-induced silencing complex (RISC). The thermodynamic properties determine the selection of the guide strand, and the passenger strand is cleaved and degraded. Perfect complementarity between guide and the target results in cleavage of the messenger RNA (mRNA). We recently described the alternatively processed AgoshRNA molecule (right side) with a ~18 bp stem and 3–5 nt loop [26]. This molecule bypasses Dicer recognition and is processed by Ago2 in between bp 10 and 11 at the 3′ side of the stem to generate an extended ~30 nt miRNA that subsequently instructs RISC for gene silencing.

Intensive investigations indicated that usually only a small percentage of chosen targets allow efficient gene silencing [28, 29]. Rational target design algorithms were subsequently

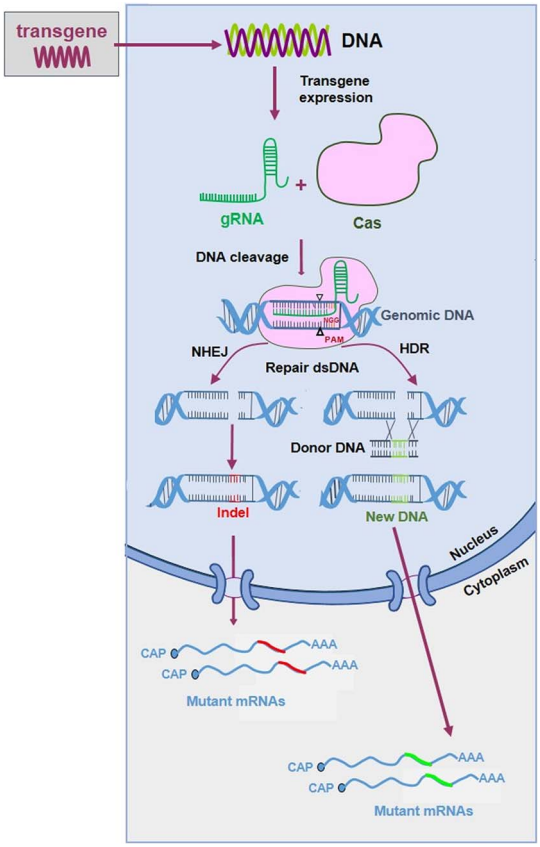

Figure 3. Schematic of the CRISPR-Cas9-mediated genome editing process. CRISPR-Cas9 requires expression of a gRNA (green line) and the Cas9 endonuclease (pink shape). The gRNA instructs Cas9 for cleavage of a complementary DNA target with an adjacent PAM sequence. Cas generates a dsDNA break that is repaired by the NHEJ or HDR pathways. See the text for further details.

developed to increase the success rate [30–34]. Another important aspect concerns the development of highly active shRNA molecules. Many factors influence the shRNA activity, including the loop sequence and thermodynamic properties of the hairpin, and guidelines for optimal shRNA design were proposed [27, 35, 36].

Specificity and off-target effects:

The efficiency and sequence specificity of RNAi make this strategy ideal to interrupt the expression of RNAs encoded by pathogenic viruses [9, 37]. RNAi can tolerate imperfections in the RNA–RNA duplex, and, as a consequence, off-target effects can be induced on non-related mRNAs. Efforts have been made to minimize this, such as careful target selection with the help of computational algorithms and optimization of the shRNA design [38–40]. Other adverse effects were reported for shRNAs. First, high-level shRNA expression can saturate the cellular RNAi machinery and cause toxicity [41, 42]. Second, either strand of the siRNA duplex can be loaded into RISC and adverse RNAi side effects can thus be induced by the passenger strand [39, 43]. In addition, shRNA expression was also reported to activate the dsRNA-induced protein kinase R (PKR)/interferon pathways [44, 45]. Strategies have been developed to minimize these problems. For instance, the use of a miRNA backbone or a reduction of the level of shRNA transcription by the use of a weak promoter can alleviate cellular toxicity issues [46, 47] and AgoshRNAs may prevent saturation of the cellular miRNA pathway as Dicer is bypassed. Importantly, the AgoshRNA design yields only a single guide strand, thus avoiding adverse effects caused by the passenger strand of a regular shRNA. Due to their smaller hairpin size, AgoshRNA may exhibit an improved safety profile concerning induction of the interferon response. Overall, AgoshRNAs exhibit some advantages by reducing off-target effects compared to regular shRNAs [26, 47], but their general value requires additional tests in different laboratories.

Delivery:

In most cases, the Ago/shRNA therapeutic is synthesized in the target cells upon delivery of a DNA vector for transient or stable gene expression [36]. Transient Ago/shRNA expression can be realized by straightforward transfection or electroporation of DNA vectors. These methods are generally safe because of the transient nature of transgene expression, but an important drawback is the poor delivery efficiency in a broad range of cell types. Viral vectors like the lentiviral vector (LV) and adeno-associated virus (AAV) vector are popular tools to deliver shRNA constructs to both dividing and non-dividing cells, including quiescent and difficult-to-transduce cells. In particular, LV-mediated delivery of antiviral genes holds promise for a durable HIV therapy because this vector integrates into the host genome, thus ensuring long-term transgene expression. These integrating vectors remain promising for durable gene therapy applications, despite the small risk of insertional mutagenesis and eventually oncogenesis. But LV has been successfully applied in the clinic to produce cancer-specific chimeric antigen receptor T lymphocytes to treat leukemia and glioblastoma [48, 49]. AAV vectors hold potential as gene delivery vectors because of their non-pathogenicity, low immunogenicity and non-integrating nature. Several gene transfer clinical trials have recently been performed with AAV vectors [50].

CRISPR-Cas9

Efficiency:

Based on differences in the components and mechanisms of action, CRISPR systems can be divided into two major systems: class 1 (type I, III, IV) requires a large complex of several effector proteins, while class 2 (type II, V, VI) only needs a single RNA-guided endonuclease (e.g. Cas9 in type II and Cas12a in type V) [51, 52]. Class 2 systems are therefore more attractive for genome editing applications. Adaptation of the bacterial CRISPR-Cas systems has facilitated application in mammalian cells, e.g. codon optimization of the Cas gene and generation of a chimeric guide RNA (gRNA) by fusion of the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) components [53, 54]. The most widely used CRISPR-Cas9 system uses the *Streptococcus pyogenes* Cas9 (SpCas9) endonuclease and a gRNA (Figure 3). A 20 nt sequence on the gRNA 5′ end is designed to be complementary to the target DNA, which contains the protospacer adjacent motif (PAM) immediately downstream of the target site (e.g. NGG for SpCas9) (Table 1). Thus, by customizing a 20 nt region of the gRNA to pair with the DNA target of interest, Cas9 can essentially target any PAM-containing genomic locus. The efficiency depends on PAM-dependent DNA binding and gRNA complementarity to the target DNA [55]. Further studies unraveled that the genomic context of the target DNA (the local GC content) and the secondary structure of the gRNA also influence the cleavage efficiency [56–59]. Tools have been designed to predict the gRNA

Table 1. In silico-guided prediction tools

In silico prediction tools	Resources
CRISPR-Design Tools	http://crispr.mit.edu/
CasFinder	http://arep.med.harvard.edu/CasFinder/
Cas-OFFinder	http://www.rgenome.net/cas-offinder/
E-CRISP	http://www.e-crisp.org/E-CRISP/
CRISPOR	http://crispor.tefor.net
Benchling CRISPR Guide Design Software	https://www.benchling.com/crispr/
CHOPCHOP	http://chopchop.cbu.uib.no
Breaking-Cas	http://bioinfogp.cnb.csic.es/tools/breakingcas/

targeting efficiency [60]. The CRISPR-Cas9 efficiency can also be improved by modification of the gRNA module: e.g. by mutation, extension or truncation. For example, extension of the gRNA duplex or mutation of the T4 stretch in the gRNA backbone can improve the efficiency by changing the gRNA structure and/or transcription rate [59].

The CRISPR-Cas9-induced double-stranded breaks (DSBs) in the cellular DNA can induce two repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Figure 3) [61, 62]. NHEJ ligates the two DSB ends, but frequently inserts or deletes nucleotides. HR repairs DSB lesions with the requirement of a homologous piece of donor DNA. The CRISPR-Cas9 system thus allows not only gene disruption but also accurate gene editing with a 'repair DNA donor'.

Specificity and off-target effects:

CRISPR-Cas9 can tolerate imperfections in the RNA–DNA duplex, and consequently, cleavage can take place at unintended off-target sites, which can sometimes be identified through in silico-guided predictions (Table 1) and genome-wide assays [63–68].

Given the complexity and large size of the human genome (~3.2 billion bp), off-target DNA cleavage poses a non-negligible risk for knockout of essential or haplo-insufficient genes or mutation of tumor-suppressor genes. These shortcomings must be overcome before CRISPR-Cas9 can be applied for somatic gene therapy applications in humans. Germline editing is not considered safe for the moment [69]. A number of approaches have been developed to reduce off-target effects, including online tools for in silico gRNA design and prediction of off-target sites [60]. gRNA optimization and Cas9 nuclease engineering have led to significant improvement of the on/off-target ratio. Truncation of the base-pairing region (spacer) of 20 nt to 17 or 18 nt was reported to increase the targeting specificity [70]. Variation in the Cas9 nuclease include the use of a paired nickase [71], fusion of the catalytically dead Cas9 to the FokI restriction enzyme [72] and development of a high-fidelity Cas9 variant (HF-Cas9) [73]. Alternatively, more specific nucleases with reduced off-target effects may be identified in natural Cas9 variants. The recently described Cas12a nuclease (formerly called Cpf1) was reported to exhibit these properties [74, 75]. Because Cas9 and other CRISPR-based endonucleases are derived from bacteria, these systems will likely elicit a host immune response. This factor of immunological risk must be considered as CRISPR-Cas9 systems advance toward clinical trials, especially for applications that require long-term Cas9 expression. Several solutions to reduce the immunogenicity risks were recently proposed [76].

Delivery:

A variety of delivery options can be considered for CRISPR-Cas9. The cargo for transient CRISPR-Cas9 delivery includes DNA vectors, Cas9 mRNA and gRNA or Cas9/gRNA ribonucleoprotein complexes [77–82]. These nucleic acids and proteins can be transfected into the cell by a variety of methods such as electroporation, cationic lipid and polymer-based transfection or lipid-based nanoparticles [83, 84]. Transient methods have a safety advantage, but will obviously not support long-term CRISPR-Cas activity that may be needed to achieve an HIV cure. Future developments in the field of nanotechnology may contribute to improving the delivery and minimizing the toxicity with highly specific cell-targeting capability. Viral vectors such as LV and AAV vectors have been widely used for the delivery of CRISPR-Cas9-encoding cassettes to a variety of cell types *in vitro* and *in vivo* [84–86]. One common problem with viral vectors is the limited packaging capacity that hinders the efficient delivery of large transgene cassettes like CRISPR-Cas9 [87–90]. For example, by delivery of the popular SpCas9/gRNA components, the LV transduction titer was significantly reduced [89]. The AAV packaging capacity of ~4.7 kb is also not sufficient for versatile CRISPR-SpCas9 applications [91, 92]. The search for alternative CRISPR-Cas systems of smaller size remains important to address these packaging problems. A smaller CRISPR-Cas9 homolog from *S. aureus* (SaCas9) could mitigate the AAV packaging problem, but this recently described system has limited efficiency and the complicated PAM sequence restricts the number of candidate target sites [57]. An alternative approach would be to minimize the modulatory elements of the CRISPR-Cas9 expression cassette, including the transcriptional promoter and the transcription termination signals.

Improvement of the new Cas12a system:

The recently discovered Cas12a system was reported to have several distinct features compared to Cas9. An increased specificity could mitigate off-target problems, and a smaller size of the cassette encoding the nuclease and matching crRNA could facilitate more efficient delivery [74, 75]. However, the Cas12a system exhibits reduced gene editing efficiency compared to Cas9 [93–95]. Inspection of the crRNA sequence raised some uncertainty about the actual 5′ and 3′ ends, and we noticed that the currently used crRNA expression approach may have some flaws [96]. This triggered us to attempt to improve the Cas12a system by focusing on optimized crRNA expression. Our polymerase (Pol) III transcription study demonstrated that specific nucleotides (+1A/G) are required for efficient production of small RNAs with a precise 5′ end and that Pol III-generated small RNAs have a 3′ U-tail of variable length [97]. These results provided useful insights on how to design more precise therapeutic RNAs. We improved the CRISPR-Cas12a system by inclusion of a self-cleaving ribozyme to create a precise crRNA 3′ end [96]. This alteration enhanced the Cas12a-mediated gene editing efficiency and allowed us more recently to move towards pro-

longed HIV inhibition experiments (unpublished). The ribozyme addition also improved the Cas12a-based gene activation platform.

Comparing the CRISPR systems, there is a pertinent difference regarding the actual DNA cleavage event. Cas9-induced DNA cleavage occurs in the PAM-proximal sequence region that is critical for gRNA binding and target DNA cleavage, while Cas12a-triggered DNA cleavage occurs in the distal region of the PAM sequence that is less critical for target binding and cleavage. This feature of Cas12a is potentially beneficial for gene inactivation because the edited DNA sequence—cleaved and repaired with the frequent inclusion of insertions or deletions (indels)—can likely be retargeted. A subsequent round of cleavage and DNA repair is likely to create a larger indel type of mutation, which is extremely beneficial for maximal HIV provirus inactivation and may prevent the generation of escape virus variants with a minimal mutation, whereas HIV genomes with a more dramatic mutation are more likely to be replication-incompetent. This concept should be tested experimentally as it could spur the route towards HIV genome inactivation and possibly a cure. We therefore anticipate that Cas12a may become a superior anti-HIV tool over Cas9.

The design of a single promoter-driven Cas9 system:

While improving Pol III-mediated AgoshRNA/crRNA expression strategies, we accidently discovered that the commonly used Pol III promoters (7SK, U6 and H1) are capable of transcribing extended mRNA transcripts that express Firefly luciferase. We subsequently demonstrated that these Pol III promoters for shRNA/gRNA expression also possess Pol II activity for mRNA transcription, but to a varying extent with H1 being the most active Pol II promoter [97, 98]. Thus, these promoters are dual-active for both polymerases II and III. We described that there is competition between these two polymerases for binding to overlapping DNA sequences of the promoter. For instance, a TATA box mutation abolished the Pol III activity and consequently enhanced the Pol II activity. Further studies are needed to map the regulatory promoter sequences that contribute to the Pol II and III activity. This should offer intriguing new possibilities to fine-tune the Pol II/III activity for the manipulation of transgene expression cassettes.

These dual-polymerase active promoter systems may be a valuable asset as they can simultaneously express a small non-coding RNA and a protein-coding mRNA. The H1 promoter seems most interesting as it exhibits abundant Pol II activity, even stronger than the standard SV40 early promoter [98]. We hypothesized that H1 can be used to express both the gRNA and Cas9 protein of the CRISPR-Cas9 system and established a single H1 promoter-driven CRISPR-Cas9 system [99]. Although this single promoter system produces less gRNA and Cas9 compared to the regular system, efficient gene editing was achieved. Most importantly, the novel cassette of reduced size should minimize the delivery problem of the viral vectors with limited packaging capacity. We indeed demonstrated that the new H1-gRNA-Cas9 system provides a significant LV titer advantage over the regular CRISPR-Cas9 system. This H1 advantage should benefit the scaled-up vector production required in preparation for future clinical trials. We expect that this novel H1-expression strategy will have similar benefits in combination with other viral expression systems such as AAV vectors. One may be able to further improve these vectors by identification of even shorter H1 promoter fragments that maintain the optimal Pol II and Pol III activity.

Key Points

- DNA/RNA modification tools such as RNAi and CRISPR-Cas9 present new possibilities for treatment of a variety of genetic and infectious diseases, including HIV/AIDS. Despite rapid optimization of these two tools, many challenges remain.
- RNAi and CRISPR-Cas9 can be used to either directly target viral components or indirectly host cell functions that fulfill a critical role during HIV-1 replication. Efficient suppression of HIV-1 replication has been described for both approaches.
- Several challenges must be overcome before the RNAi and CRISPR-Cas therapeutics can fulfill their clinical potential. The issues involved include ways to prevent or minimize possible off-target effects and optimization of the delivery strategies in order to reduce the risk of viral escape.

Author's perspective

Comparing the antiviral RNAi and CRISPR-Cas approaches, the latter method has the unique ability to directly target the integrated HIV provirus, which is the main cause of viral persistence under cART therapy and the viral rebound that inevitably occurs when therapy is stopped. Impressive results have been reported concerning the complete inactivation of infectious HIV in a simple, but powerful *in vitro* cell culture model [100] and *in vivo* in a small subset of CRISPR-treated humanized mice [22]. But at least two major challenges lie ahead of us. One, how can we target all or at least a significant fraction of the HIV-reservoir cells in an infected individual? This requires a more complete description of the tissues and cell types in which HIV can hide. The reservoir includes resting T cells and the recently described CD32a-positive T cells [101], but likely additional cell types. Second, it seems unsafe to deliver the functional CRISPR machinery by a vector system that persists and continues to produce the foreign endonuclease. Although CRISPR acts in a sequence-specific manner, it could over time start to modify non-HIV off-target sites in the human genome. Such genetic changes can eventually trigger oncogenesis. Thus, it would seem important to focus on transient CRISPR systems and to check if the same antiviral efficiency can be achieved. At the end, anti-HIV efficacy and safety can hopefully go hand in hand to deliver the first man-made HIV cure.

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